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[54] FAST PROCESS FOR DETECTING AND/OR IDENTIFYING A SINGLE BASE ON NUCLEIC ACID SEQUENCE AND ITS APPLICATIONS

[57] This process is characteristic in that:
(1) the sequence on which the base to be identified is located is made hybrid with a nucleotide of adequate length;
(2) the synthesis of the complementary strand of the hybrid obtained in (1) is started, said nucleotide acting as a trigger, in the presence of:
- a polymerase without 3'5' exonucleasis action and
- at least one modified nucleotide base;
(3) the incorporated blocking nucleotide base is detected by any suitable means.
Application: diagnosis of genetic diseases.

This invention concerns a process for detecting and/or identifying a single base on a nucleic acid sequence and its applications, specifically for diagnosis of genetic diseases and for controlling hybridization.

Hybridization of nucleic acid has been used to study the identity and to determine the presence of nucleic acids. Hybridization is based on the pairing of complementary bases. When complementary single-strand nucleic acids are incubated together, these single-strand nucleic acids pair up to form a double-strand hybrid molecule. The capability of single-strand DNA or RNA to form a structure with a complementary nucleic acid sequence is used as a method of analysis and diagnosis. The availability of radioactive triphosphate nucleosides with significant specific activity and the marking of the DNA at ^{32}P in the presence of enzymes with a polymerase function, such as T_4 kinase, has made it possible to identify, isolate, and characterize many nucleic acid sequences of biological interest.

Hybridization is an important criterion in detecting the presence of specific nucleic acid sequences, for example:

- in human or animal genetic diseases where hereditary modification of the genetic makeup has morbid consequences (via insertion, deletion, or periodic mutation of a specific sequence);
- in cancers where rearrangements of the genomic DNA are observed;
- in infections where the presence of foreign genomes is detected, such as those of microorganisms (bacteria, fungi, and viruses, for example);
- and to identify individuals in general:
- in forensic medicine (paternity and filiation, for

resistant to digestion under specific conditions;
(c) digestion of the hybrid by an exonuclease under such conditions that the double-strand is progressively digested from the end of the probe, unless that end has been joined with said nucleotide derivative;
(d) elimination of portions of the probe that are no longer hybridized to the nucleic acid chain;
(e) and detection of a mutation of the specific nucleotide base in the target sequence by detection of the presence or absence of the probe after digestion.

In addition to the probe, this process involves, specifically, the use of a nucleotide derivative with specific properties, and therefore still involves a number of stages. In addition, for its implementation it requires immobilization of the nucleic acid on a membrane, and also marking (of the probe or of the nucleotide derivative).

Therefore the purpose of this invention is to provide a process for identifying a specific nucleotide base that is easy and fast to use, which better responds to practical requirements than the processes of the prior art, specifically in that the process pursuant to the invention does not require a complex operational protocol, i.e. it requires no immobilization of the DNA nor marking of the probe, and applies to detecting specific nucleic acid sequences, specifically in genetic diseases, cancers, infections, and the identification of individual human, animal or plant specimens.

The object of this invention is a process for detecting and/or identifying a specific nucleotide base present on a nucleic acid sequence, characteristic in that:
(1) the sequence on which the base to be identified is

located is made hybrid with a nucleotide of adequate length to allow for correct hybridization, regardless of the reaction temperature, said nucleotide hybridizing with the target sequence, so that its 3' end is adjacent to the specific nucleotide base to be detected and/or identified;

(2) the synthesis of the complementary strand of the hybrid obtained in (1) is started, said nucleotide acting as a trigger, in the presence of:

- a polymerase without 3'5' exonucleasis action, and
- at least one modified nucleotide base, so that it can be incorporated into the extension product of the trigger, said incorporation blocking the elongation of said extension product;

(3) the incorporated blocking nucleotide base is detected by any suitable means, said detection allowing for identification of the specific complementary nucleotide base located on the target sequence to be analyzed.

For purposes of this invention, a nucleotide means both an oligonucleotide that has 10 to 50 bases, and a nucleotide that may have more than one hundred bases.

Pursuant to one advantageous manner of implementing this process, the blocking nucleotide bases are dideoxynucleotides.

Pursuant to another advantageous manner of implementing this process, said blocking nucleotide bases are suitably marked, specifically by a marker selected from the group that includes radioactive substances, enzymes, fluorescent or chemoluminescent chromophoric chemical products, and appropriate antibodies.

Pursuant to one advantageous form of this manner of implementing the process, the marker is identical to or different from each of the blocking nucleotide bases.

Pursuant to one modality of this form,

a short oligonucleotide probe of 17 to 24 nucleotides can be synthesized, the center of which coincides with the mutation that one wishes to detect. By selecting suitable hybridization and rinsing conditions (specific for each system), hybridization by means of marked oligonucleotides can be achieved only in case of perfect equivalence (the difference of a single nucleotide, particularly at the site of the mutation, results in destabilization of the hybridization).

However, these various methods all have a certain number of disadvantages:

- the temperature conditions are difficult to master to achieve suitable hybridization;
- the mandatory presence of a restriction site may be required;
- the nucleic acid is immobilized on a membrane (Southern blot).

The U.S. patent AMERSHAM no. 4,656,127 did permit improvement of some of these disadvantages, specifically in that it permits detection of a mutation at a position that does not provide a site for cleavage by a restriction enzyme.

This U.S. patent no. 4,656,127 describes a method for detecting the mutation of a specific nucleotide base in a target fragment of nucleic acid (DNA or RNA) by:
(a) hybridization of a probe with the target sequence to form a nucleic acid hybrid, in which one end of the probe is made hybrid in a manner adjacent to the specific nucleotide base;
(b) mixing the hybrid with a nucleotide derivative under conditions suitable for elongating the probe, to permit junction of the nucleotide derivative and the end of the probe, only if the specific base in the target sequence is (or is not) the mutation to be detected, a probe associated with that nucleotide derivative being

example), and

- in the farm produce sector (plants and health inspection, for example).

However, hybridization as a diagnostic tool can be limited by the difficulty of implementing it (cumbersome techniques) or by the lack of specificity of the hybridization (operative protocol).

In fact, chemical study of hybridization has clarified the effects of the concentration of each of the strands of nucleic acids involved, as well as their length, composition in bases, the temperature, pH level, ionic strength, and the viscosity of the medium.

Temperature is particularly critical, and must remain lower than the fusion temperature (Tm: temperature at which 50% of the sequences are in double-strand form). In solution, the optimum hybridization temperature is 25°C below Tm for a probe of 150 nucleotides, and slightly lower for shorter probes.

Thus, to detect a mutation involving a single base, depending on the case generally two types of probes can be used: nucleic acid probes called long probes, generally over 150 nucleotides, or nucleic acid probes called short probes, generally between 17 and 24 nucleotides. If the mutation occurs at a site recognized specifically by an enzyme called a restriction enzyme, the Southern blot technique can be used. This technique includes stages of isolating the DNA, digestion by the restriction enzyme, electrophoresis on gel, transfer onto a membrane, and hybridization by means of a long probe involving the region of the mutation; after washing and autoradiography, analysis of the size of the fragments obtained permits confirmation or invalidation of the presence of the mutation. This very cumbersome process requires that the mutation involve a restriction site. If this is not the case,

if the four blocking bases are marked by means of different markers, the four blocking nucleotides are advantageously detected at the same time.

Pursuant to another advantageous manner of implementing this process, if the four blocking bases are marked identically, or if they are unmarked, detection of the four blocking nucleotides occurs successively and/or separately.

Pursuant to one advantageous form of this manner of implementing the process, the pyrophosphate formed during the polymerization reaction is detected.

In fact, the polymerization reaction generates the production of a pyrophosphate, as follows:
matrix - trigger + dNTP ---> matrix - (trigger + dNMP) + PP_i.

By measuring the pyrophosphate in each reaction tube, it is possible to determine for which of the bases a polymerization reaction occurred.

Pursuant to another advantageous form of this manner of implementing the process, each marked nucleotide base is detected.

In fact, each reaction tube contains, in this case, a single marked nucleotide base; the other three are unmarked. Measurement of the marked base (via fluorescence, radioactivity, etc.) makes it possible to determine for which of the bases a polymerization reaction has occurred, as the unincorporated marked bases are eliminated through washing.

One specific advantage of the process pursuant to the invention is that it defines the operative conditions independently of the nucleotide base to be identified, and does not require immobilization of the nucleic acid on a membrane.

Another object of this invention is a ready-to-use diagnostic kit or case for implementation of the process pursuant to the invention, characteristic in

(1/200 [a] and 1/400 [b]).

As noted above, this invention is not in any way limited to the modes of implementation, realization and application that have been described in greater detail; rather, the invention includes all the variations that may occur to the minds of technical experts in this field, without exceeding the framework or scope of this invention.

d) Washing the excess fluorescent dideoxynucleotides:

* place the samples on a Sephadex G50 column,
* recover the eluate.

e) Detection

Detection is performed for each sample after electrophoretic migration and excitation by a source such as a laser. The signal is analyzed by a fluorometer.

The curves [a] (control) and [b] (patient), as shown in figure 1, are obtained, which makes it possible to detect the incorporation of ddATP in [a] for a healthy subject, at the position corresponding to a potential mutation, and the incorporation of ddTTP for the homozygous individual in [b].

Example 2: Microsequencing of a base on a nucleic acid sequence (DNA of the bacteriophage M13 mp8).

a) Initial material

* Single-strand DNA of bacteriophage M13 mp8
* Synthetic oligonucleotide called "Universal Primer", with sequence: 3' TGACCGGCAGCAAAATG 5'

The first base incorporated on the trigger in the direction 5'-->3' is a G.

b) Protocol .

The protocol is the same as the detection protocol for mutation at a given point starting with stage [c] of example 1. Stages [a] and [b] are inapplicable, since the DNA of the phage M13 is not contaminated by oligonucleotides.

As a variant, the protocol is as follows:

- 3 µg of M13 single-strand DNA
- 15 ng of universal primer oligonucleotide
- 7 µl of 5X Sequenase aliquot
- H_2O qsp 22 µl.

Detection shows the incorporation on the primer of a dideoxy GTP, which corresponds to the expected result, as illustrated in figure 2, which shows the results obtained with two different dilutions of the ddNTP

b) Elimination of the excess cold dideoxynucleotides

The Centricon 3 or 10 (Amicon) microseparation systems are used which, by filtration on membrane accelerated by centrifuging, make it possible to retain molecular specimens for molecular weights above 3,000 or 10,000 Daltons (for example: one nucleotide is 330 D and one oligonucleotide synthesized from 20 parents is 6,600 D).

- place the reaction product resulting from a) in a "Centricon" 3 or 10 and dilute, if necessary,
- for less than 5,000 g, centrifuge by following the manufacturer's instructions, so that a minimum amount is recovered,
- invert the "Centricon" system,
- centrifuge to recover the reaction volume,
- bring the volume obtained to 12 µl.

c) Microsequencing (process pursuant to the invention)

* For each tube of DNA, add to the 12 µl:
- 15 ng of oligonucleotide 5' CATGGTGCACCTGACTCCTG 3' (OA), corresponding to the sequence that stops at the base adjacent to the position of the mutation
- 7 µl of Sequenase 5X aliquot as defined in a) above and follow the procedure as in a); then
* prepare the reaction aliquot set up for each sample with:
- 2.5 µl DTT 0.1 M
- 1 µl of a 1/400 dilution of a mixture of fluorescent ddNTP (ddTTP*: 112 µM; ddGTP*: 1.12 µM; ddATP*: 3.36 µM; ddCTP*: 8.96 µM). The fluorescent dideoxynucleotides are sold by DuPont (Genesis™ 2000 DNA Analysis System);
- H_2O qsp 6.5 µl
- Sequenase 1 µl (3 units)
* remove the tubes from the water bath, centrifuge quickly,
* add the reaction aliquot, mix,
* maintain the water bath at 37°C for 5 minutes,
* remove the tubes and place them in ice.

Example 1 - diagnosis of drepanocytosis via the process pursuant to the invention (measuring fluorescent dideoxynucleotides).

Drepanocytosis or sickle-cell anemia is caused by a mutation in one of the exons of the β gene coding for the β chain of hemoglobin (Hb). This mutation, which consists of substituting a thymine (T) for an adenine (A), modifies the GAG codon, translated into a glutamic acid (in the G position) in normal Hb, into a GTG codon that is translated into a valine in abnormal Hb (HbS). The DNA used in this example corresponds to an amplified single-strand DNA sequence.

a) Blocking the amplification triggers.

* for each DNA matrix, mix in a microcentrifuge tube:
- 100 ng of DNA (amplified single-strand)
- 7 µl of Sequenase 5X aliquot (= Tris-HCl pH 7.5, 200 mM; NaCl, 250 mM; $MgCl_2$, 100 mM)
- H_2O qsp 22 µl
* agitate using a vortex and centrifuge rapidly
* incubate in a water bath at 95°C for 2 minutes
* remove quickly and place in a water bath at 37°C for 10 minutes.
* prepare the reaction aliquot for a DNA sample, composed of:
- 2.5 µl DTT 0.1 M
- 1 µl of a mixture of ddNTP, diluted to 1/400 (ddTTP: 112 µM; ddGTP: 1.12 µM; ddATP: 3.36 µM; ddCTP: 8.96 µM); and
- H_2O qsp 6.5 µl
- Sequenase 1 µl (3 units)
* remove the tubes from the water bath and centrifuge quickly,
* add the reaction aliquot and mix,
* place in the water bath at 37°C for 5 minutes,
* remove the tubes and place them in ice.

that in addition to suitable quantities of reagents and aliquots appropriate for implementing the process, it contains:

- suitable quantities of a nucleotide (acting as a trigger) capable of hybridizing with the target sequence so that its 3' end is adjacent to the specific nucleotide base to be detected;
- suitable quantities of four modified nucleotide bases so that they can be incorporated into the extension product of the trigger while blocking the elongation of said extension product; and
- suitable quantities of a polymerase without exonucleasis 3'5' action.

Pursuant to one advantageous manner of realizing said kit or case, the modified nucleotide bases are dideoxynucleotides (ddTTP, ddGTP, ddATP, ddCTP).

Pursuant to another advantageous manner of realizing said kit or case, the modified nucleotide bases are marked in a suitable manner, specifically by a marker selected from the group that includes radioactive substances, enzymes, fluorescent or chemoluminescent chromophoric chemical substances, and suitable antibodies.

Pursuant to another advantageous manner of realizing said kit or case, it also includes reagents suitable for measuring the pyrophosphate.

In addition to the preceding forms, the invention also includes other forms that will be apparent from the following description.

The invention will be better understood through the additional description below, which refers to examples of implementation of the process that is the object of this invention.

However, it must be clearly noted that these examples are given solely for purposes of illustration of the object of the invention, and do not in any way constitute a limitation of the invention.

characteristic in that the modified nucleotide bases are dideoxynucleotides.

11) Kit or case pursuant to claim 9 or claim 10, characteristic in that the modified nucleotide bases are suitably marked, specifically by means of a marker selected from the group that includes radioactive substances, enzymes, fluorescent or chemoluminescent chromophoric chemical substances, and suitable antibodies.

12) Kit or case pursuant to any one of claims 9 through 11, characteristic in that it also contains suitable reagents for measuring the pyrophosphate.

13) Application of the process pursuant to any one of claims 1 through 8, for detecting the presence of specific nucleic acid sequences related to diseases, such as genetic diseases or cancers, infection, or for identifying human, animal, or plant specimens.

5) Process pursuant to claim 4, characteristic in that if the four blocking bases are marked with different markers, detection of the four blocking nucleotides is advantageously simultaneous.

6) Process pursuant to any one of claims 1 through 4, characteristic in that if the four blocking bases are marked identically, or if they are unmarked, detection of the four blocking nucleotides occurs successively and/or separately.

7) Process pursuant to claim 6, characteristic in that the pyrophosphate formed during the polymerization reaction is measured appropriately, said measurement of the pyrophosphate making it possible to determine for which of the bases a polymerization reaction occurred.

8) Process pursuant to claim 6, characteristic in that each marked blocking nucleotide is detected.

9) Ready-to-use diagnostic kit or case for implementing the process pursuant to any one of claims 1 through 8, characteristic in that in addition to suitable quantities of reagents and aliquots appropriate for implementing the process, it contains

- suitable quantities of a nucleotide, acting as a trigger, capable of hybridizing with the target sequence so that its 3' end is adjacent to the specific nucleotide base to be detected;
- suitable quantities of four modified nucleotide bases so that they can be incorporated into the extension product of the trigger while blocking the elongation of said extension product; and
- suitable quantities of a polymerase without exonucleasis 3'5' action.

10) Kit or case pursuant to claim 9,

CLAIMS

1) Process for detecting and/or identifying a specific nucleotide base located on a nucleic acid sequence, characteristic in that:

(1) the sequence on which the base to be identified is located is made hybrid with a nucleotide of adequate length to allow for correct hybridization, regardless of the reaction temperature, said nucleotide hybridizing with the target sequence, so that its 3' end is adjacent to the specific nucleotide base to be detected and/or identified;

(2) the synthesis of the complementary strand of the hybrid obtained in (1) is started, said nucleotide acting as a trigger, in the presence of:

- a polymerase without 3'5' exonucleasis action, and
- at least one modified nucleotide base, so that it can be incorporated into the extension product of the trigger, said incorporation blocking the elongation of said extension product;

(3) the incorporated blocking nucleotide base is detected by any suitable means, said detection allowing for identification of the specific complementary nucleotide base located on the target sequence to be analyzed.

2) Process pursuant to claim 1, characteristic in that the blocking nucleotide bases are dideoxynucleotides.

3) Process pursuant to claim 1 or claim 2, characteristic in that said blocking nucleotide bases are suitably marked, specifically by means of a marker selected from the group that includes radioactive substances, enzymes, fluorescent or chemoluminescent chromophoric chemical substances, and suitable antibodies.

4) Process pursuant to claim 3, characteristic in that the marker is identical or different for each of the blocking nucleotide bases.

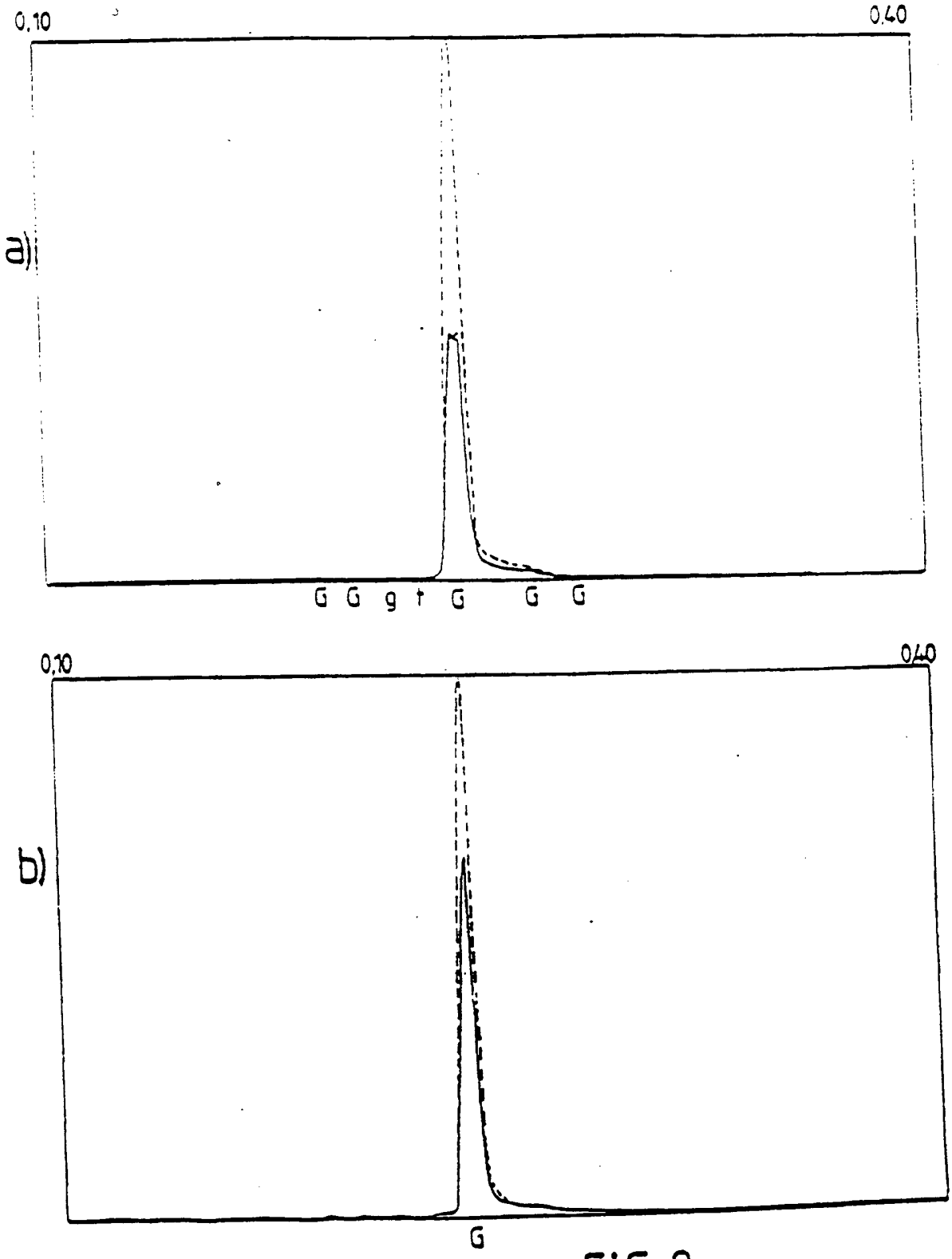

FIG.2

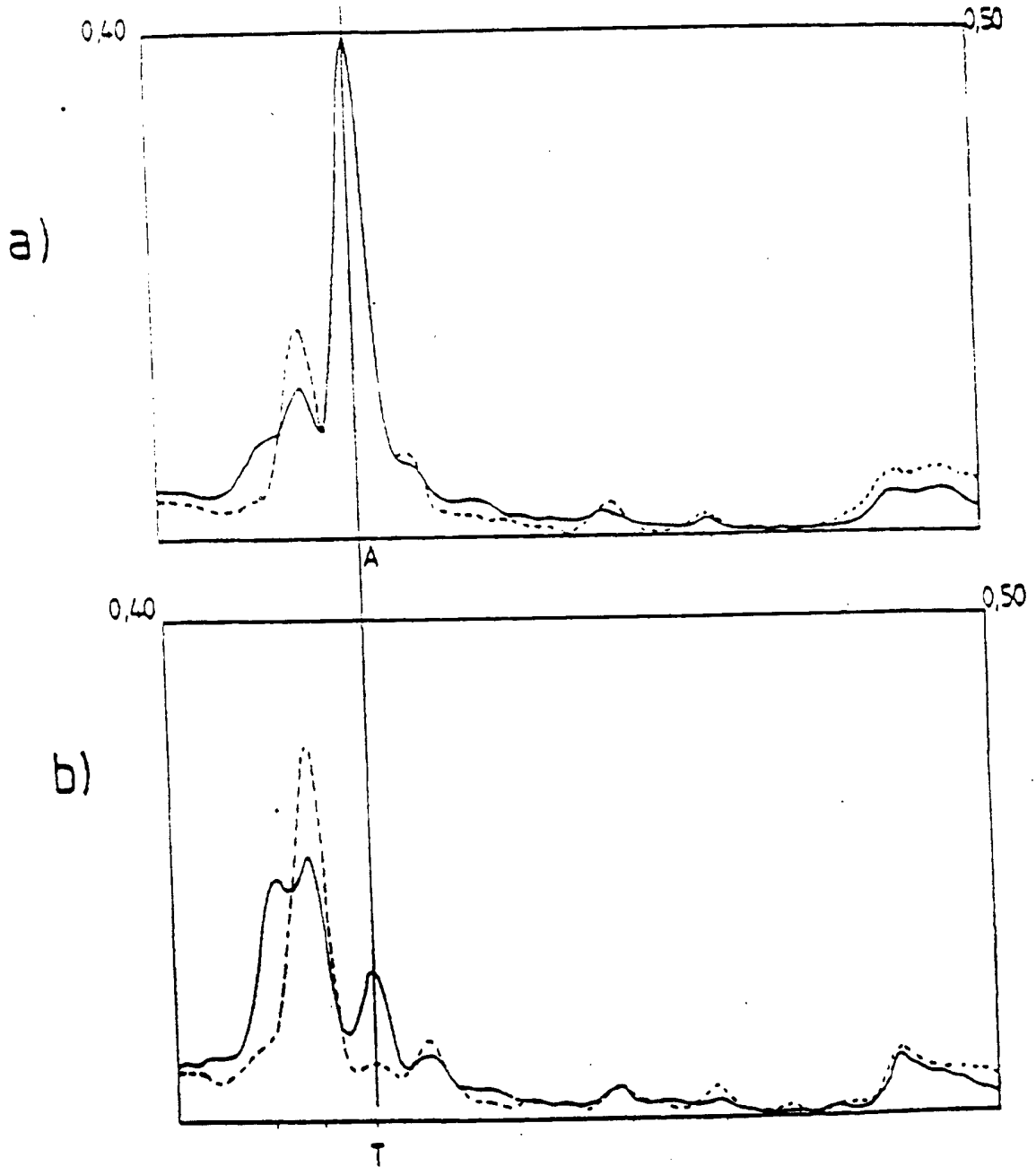

FIG.1